REMARKS

1. The Amendments, the Support Therefor, and Basis for Entry

The foregoing amendments are made with the understanding that the amendments filed in the Response of 11 December 2002 were not entered. Four claims (6, 19, 32, and 45) have been canceled, four new claims (53-56) have been added, and claims 1, 7, 9, 11, 14, 20, 22, 24, 27, 33, 35, 37, 40, 46, 48, and 50 have been amended to leave claims 1-5, 7-18, 20-31, 33-44 and 46-56 in the application. No new matter has been added by the amendments or new claims.

Regarding support for the amendments, in claims 1, 14, 27, 40, support for "at least 40 base pairs" can be found at page 7 line 21; "intercalating dye", page 8 line 25; and "temperature", page 8 line 27 onward. In claims 9, 22, 35, 48, support for "salt concentration less than 200 mM" can be found at page 13 lines 1-6.

2. Reconsideration of Rejections

Reconsideration of the rejections is requested on the basis of the comments noted below. In some cases, these comments clarify and amplify those made in the prior Response of 11 December 2002. It is understood that the USPTO did not find some of these arguments convincing for the following reason noted in the 31 December 2002 Advisory Action:

> Specifically, the issue is whether the term "solid surface" as used by the ordinary artisan, encompasses polyacrylamide gels. Specific evidence showing that such a gel is treated as an equivalent solid surface was presented in the final rejection.

However, the prior Response of 11 December 2002 specifically rebutted this assertion, and it is not understood why the Applicant's comments – which were supported by a Declaration of a recognized expert – were not found convincing. In any further Office Action, if there is disagreement with the Applicant's assertions, kindly provide more detailed comment so that the Applicant may better determine how to proceed.[1]

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[1] See MPEP 707.07(f), Answer All Material Traversed ("Where the applicant traverses any rejection, the examiner should, if he or she repeats the rejection, take note of the applicant's argument and answer the substance of it"); also see Examiner Notes for PTO form paragraphs 7.37 and 7.38 (as reproduced in MPEP 707.07), which require that all relevant arguments by the Applicant be addressed, as well as

While certain comments below clarify and amplify those made in the prior Response of 11 December 2002, please note that new comments are made as well. As per MPEP 2144.08 (section entitled "Reconsider All Evidence And Clearly Communicate Findings And Conclusions"), kindly make a thorough review of all arguments and evidence, without prejudice arising from the prior rejections made in this case. As discussed below, both objective evidence and expert opinion support the allowability of the present claims, and careful reconsideration of the rejections is requested.

3. Sections 1-2 of the Office Action: Rejection of Claims 1-6, 8, 10-19, 21, 23-32, 34, 36-45, 47, and 49-52 under 35 USC §103(a) in view of *Drobyshev et al* (Gene (1997) 188:45-52) and U.S. Patent 6,174,670 to *Wittwer*

3.a. Comparison of Invention vs. Cited References

The present invention is concerned with polymorphism scoring methods that are simple and robust enough for automated high volume screening. Previously reported methods of allele discrimination on a solid surface suffered from low signal strength, high background and poor discrimination between alleles. Low signal strength meant that long exposure times were necessary in order to detect signal bound to the surface. These long exposure times dictated that the solid phase hybridization methods could only be used in a static fashion to determine hybridization at a particular temperature. Dynamic, real-time measurement of hybridization was not possible.

Both *Drobyshev et al* and *Wittwer* can be seen as alternative approaches to these previous methods, and both are aimed at achieving greater sensitivity than could be achieved with solid surface hybridization methods for allele discrimination. *Drobyshev et al* teaches the hybridization of fragmented RNA molecules to oligonucleotides immobilized within polyacrylamide gel pads. The RNA molecules are fluorescently labeled and the amount of label bound to the immobilized

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MPEP 706.07 under "Statement of Grounds" ("the final rejection . . . also should include a rebuttal of any arguments raised in the applicant's reply").

oligonucleotide is measured using fluorescence microscopy to produce a melting curve. To provide a standard reference Td, an oligonucleotide may be used instead of an RNA molecule. The increased binding capacity and hybridization characteristics of oligonucleotides immobilized in three dimensions within the gel lead to dramatic improvements in sensitivity.

Wittwer teaches real-time monitoring of hybridization between nucleic acid strands in a PCR reaction using a variety of formats including double stranded DNA dyes, pairs of FRET labeled oligonucleotide probes and single FRET labeled probes with FRET labeled target sequence. As amplification by PCR produces large amounts of target nucleic acid, all of which is assayed, the requirement for sensitivity in monitoring the hybridization of this target is reduced.

3.b. The Cited References Do Not Disclose or Suggest Solid-Phase Surface Nucleic Acid Hybridization

Neither *Wittwer* or *Drobyshev et al*, whether taken alone or in combination, describes or suggests the claimed ***solid-phase*** nucleic acid hybridization feature of "a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation bound to a solid surface."

Initially, it is apparent that the references do not disclose the claimed arrangement. *Wittwer* is entirely concerned with PCR reactions in solution and there is no teaching of the immobilization of any of the components of these reactions. *Drobyshev et al* then employs an array of 10mer oligonucleotides which are immobilized *within* a polyacrylamide gel. Note that *Drobyshev et al* clearly states on page 48 that the "[p]olyacrylamide gel provides a stable three dimensional support for immobilized oligonucleotides" (first full paragraph). The immobilized oligonucleotides are thus arranged *within* the body of the gel pad *in a three dimensional arrangement*. Oligonucleotides immobilized in this way are not bound to a "surface" as that term is commonly understood to an ordinary artisan. Further evidence that an ordinary artisan would not regard a gel to be a "solid surface" is discussed at Sections 3.d-3.e later in this Response.

Additionally, the references in no way suggest that any advantage would be obtained by binding the oligonucleotides to a solid surface. The three dimensional arrangement of oligonucleotides is essential to the working of the *Drobyshev et al* method and is not simply an

equivalent to a single layer of oligonucleotides bound to a solid surface. For example, page 48 of *Drobyshev et al* states (at first column, midway down):

> The polyacrylamide gel provides more than 100 times greater capacity for three dimensional immobilization of oligonucleotides than does a two dimension glass surface. The high concentration of immobilized oligonucleotides facilitates the discrimination of mismatch duplexes and enhances the sensitivity of measurements on the microchips.

The polyacrylamide gel is thus used in *Drobyshev et al precisely because it is not a "surface"*, in order to immobilize oligonucleotides in a three dimensional array to increase binding capacity and thus sensitivity. This further demonstrates that an ordinary artisan would not modify *Drobyshev et al* to meet the claimed invention; see MPEP 2143.01 (subsection entitled "The Proposed Modification Cannot Render The Prior Art Unsatisfactory For Its Intended Purpose").

In the molecular hybridizations described in *Drobyshev et al*, the gel pads provide a three dimensional support for the immobilized oligonucleotides: it is stated several times and is, in fact, crucial to the method, as it provides an improvement in sensitivity of two orders of magnitude. A body that has an embedded three dimensional array of immobilized oligonucleotides is not a "surface" and would not be so considered by an ordinary artisan. Oligonucleotides arranged within the body of a gel pad are not "bound to a surface" in the same way that a submarine submerged under the sea is not "at the surface", whereas a boat floating on the sea is "at the surface".[2]

The combined teachings of *Drobyshev et al* and *Wittwer* also fail to offer a solution to the fundamental problem of low sensitivity in monitoring hybridization on a solid surface because hybridization on a solid surface has entirely different physico-chemical properties from hybridization in solution or within the body of a gel. Nucleic acid molecules bound to a solid surface are tightly packed in the same orientation, and each molecule interacts with its neighbors. These interactions may affect the hybridization, for example causing high background: signal

[2] It is also somwhat relevant that a gel represents a phase intermediate between solid and liquid. Since a gel is neither solid nor liquid, an ordinary artisan would not consider the gel pads of *Drobyshev et al* to fulfill either of the requirements of the present claims that the oligonucleotides be bound to (1) a surface (2) of a solid.

ratios. In a liquid, however, the nucleic acid molecules are not aligned and are well spaced, minimizing inter-molecular interactions. Hybridization in a gel phase has properties very similar to that of a liquid phase. *Drobyshev et al* itself specifically states that "hybridization within the gel looks more like a liquid phase than a solid phase reaction" (page 48, 4th sentence of section 2.3). Neither *Drobyshev et al* nor *Wittwer* therefore addresses the problems intrinsic in performing and monitoring hybridization on a solid surface, and the skilled person's expectation that solid phase hybridization cannot be monitored in a sufficiently sensitive manner for dynamic (i.e. real-time) reading is unaltered by the combined teaching of these disclosures.

The combined teaching of *Drobyshev et al* and *Wittwer* is therefore deficient in failing to teach "a single DNA strand bound to a solid surface". Any combination of these teachings by a person of ordinary skill would, in fact, result in nucleic acid molecules were either immobilized within a gel or free in solution. Neither of these possibilities is encompassed by the present claims.

3.c. The Art As A Whole Teaches Away From The Recited Arrangement

In addition, this Response is accompanied by evidence from the field of nucleic acid hybridization demonstrating that an artisan skilled in this field would be aware of significant technical differences between hybridization on a solid surface (such as glass), and hybridization within a polyacrylamide gel; and that given these differences, an ordinary artisan would not find it advantageous to combine the cited references to obtain the claimed invention.

Initially, the differences between a two-dimensional solid surface and a three-dimensional gel are summarized on page 344 of *Kochinsky & Mirzabekov* (2002) Human Mutation 19: 343-360:

> Commercially available arrays are usually manufactured by immobilizing DNA probes on nylon membrane.... or glass.... The analysis is performed by hybridization of liquid sample to probes arranged in a two-dimensional monolayer. Although it proved to be advantageous for high throughput applications, the two dimensional arrangement of probes has its limitations: enzymes do not always work on flat microchips; the concentration of probe and therefore sensitivity are relatively low; the use of short probes with high discrimination capacity is difficult; technology is rather complex and costly. Therefore we developed microarrays of three dimensional gel elements based on an alternative approach to study interactions between immobilised and dissolved molecules.

This is confirmed in *Livshits & Mirzabekov* (1996) Biophysical J. 71 2795-2801, which states:

> Such a three dimensional immobilization (relative to two dimensional (2D) immobilization on a glass or filter surface) has been shown.... to be advantageous....

There are therefore various drawbacks associated with hybridization on two-dimensional surfaces, particularly in terms of sensitivity and discrimination, which the Mirzabekov group have addressed by replacing the solid surface with a three-dimensional gel element. In particular, the binding capacity of the gel is reported to be higher than that of a solid surface (see page 344 col 2);

> Tethering the molecules within a three dimensional gel significantly increases, 10^2 to 10^3 times, increases the immobilization capacity as compared to the equivalent glass surface.

Furthermore, the physico-chemical properties of hybridization within a gel pad are also advantageous over hybridization on a surface;

> Interactions of macromolecules with gel-immobilized compounds occur in the surrounding homogeneous liquid and the physics of these processes is more similar to water solution than heterophase interactions with a glass immobilized compounds.

The distinct properties of surface phase hybridization relative to liquid phase is discussed in more detail in *Peterson et al*, J. Am. Chem. Soc. (2002) 124 14601-14607 (enclosed). Differences between hybridization on solid surfaces and three-dimensional gels, in particular with regard to sensitivity and discrimination, are also noted in *Livshits et al* (1994) J. Biomol. Struct. Dyn. 11 783-795:

> This dependence [of thermostability on concentration] is specific for oligonucleotides immobilized in the gel volume (3-D-immobilization) rather than on a flat surface of a filter or glass (2-D-immobilisation).

> The gel-immobilized oligonucleotide matrix provides also a higher capacity for immobilization and therefore a higher sensitivity of measurements, resulting in a higher discrimination power for identification of perfect duplexes as compared with matrixes of oligonucleotides immobilized on a surface.

The fundamental differences between 3D gel pads and 2D surfaces are confirmed in the enclosed declaration from Professor Andrei Mirzabekov of the Engelhardt Institute in Moscow (NOTE that it was Mirzabekov's group which produced the *Drobyshev et al* (1997) paper in issue). A list of papers on the PubMed database authored by Professor Mirzabekov is attached. The Kwok Declaration, submitted in the prior Response and included with this Response for the convenience

of the Examiner, further states why an ordinary artisan would not attain the claimed invention from a review of the cited references and any advantages stated therein.

In summary, prior to the present invention, people in the field were aware of various drawbacks associated with two-dimensional surface hybridization. The Mirzabekov group and others addressed these drawbacks by developing three-dimensional gels containing immobilized probe sequences. In contrast, the present inventors persisted with solid surface hybridization and developed an improved method which employs hybridization on a two-dimensional surface, but which does not suffer the drawbacks of previous methods. In particular, the sensitivity and discrimination of this method allows real-time allele discrimination on a solid surface. The ability to use a solid surface for allele discrimination considerably simplifies the production of reagents for these methods, reducing costs and facilitating high throughput.

In the light of this evidence, it is clear that hybridization at a solid surface is very different from hybridization within a gel pad; that an artisan of ordinary skill in this field would understand that a gel pad is not a solid surface; and that an artisan of ordinary skill would not be motivated to modify *Drobyshev et al*, or in fact any references of record, to utilize surface hybridization as recited.

3.d. The Plain Meaning of "Surface" Does Not Encompass The Interior of a Gel

Where a claim term is an everyday word which is not assigned a special or limited meaning by the specification, a dictionary should be used to determine the meaning that should be given to the term. *Optical Disc Corp. v. Del Mar Avionics*, 54 USPQ2d 1289, 1295 (Fed. Cir. 2000); *Vanguard Products Corp. v. Parker Hannifin Corp.*, 57 USPQ2d 1087, 1089 (Fed. Cir. 2000). Here, a "surface" is defined as:

- the outer or top part or layer of something
  (Cambridge International Dictionary of English, http://dictionary.cambridge.org/define.asp?key=surface*1+0)
- the exterior or upper boundary of an object or body
  (Merriam Webster's Collegiate Dictionary, 10th Edition, http://www.m-w.com/cgi-bin/dictionary?book=Dictionary&va=surface)

- 1a. The outer or the topmost boundary of an object. b. A material layer constituting such a boundary.

  (The American Heritage Dictionary of the English Language, http://www.bartleby.com/61/29/S0912900.html)

- the outer part or external aspect of an object

  (Dorland's Illustrated Medical Dictionary, http://www.mercksource.com)

- The exterior part of anything that has length and breadth; one of the limits that bound a solid, especially. The upper face; superficies; the outside.

  (The On-line Medical Dictionary, http://cancerweb.ncl.ac.uk/cgi-bin/omd?surface)

Thus, the plain meaning of "surface" does not encompass the interior of a gel, as in *Drobyshev et al.* This is confirmed by the Kwok declaration, which notes at Section 4, Page 2 that

> an ordinarily skilled artisan would not consider that these gel immobilized oligonucleotides are 'bound to a solid surface'.[3]

3.e. <u>The Cited US02/0109841 Does Not Support The Assertion That The Interior of a Gel is a "Surface"</u>

The Examiner has cited US02/0109841 as evidence that the term "solid surface" may encompass a polyacrylamide gel. However, with all respect, this assertion is flawed in two respects.

First, the reference is nonanalogous art, and thus its contents and terminology do not speak to what one of ordinary skill *in the art of the present invention* would understand "surface" to encompass. US02/0109841 relates to analytical instrumentation for identifying substances, more specifically a scanning spectrophotometer for use in detecting fluorescence in liquid samples to identify compounds therein. The field of spectrophotometry (and more generally analytical instrumentation) is an entirely different field from that of the present invention. This is in part demonstrated by the fact that the US search classification (356/318) of US02/0109841 is completely different from that of the other primary art cited in this case (classification 435/6).

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[3] Since the Applicant has now submitted ***two*** expert Declarations testifying to the unobviousness of the claimed invention – one of these, from Mirzabekov, being from the same group that produced *Drobyshev et al.* – kindly grant their contents close attention (particularly since these are difficult and expensive to obtain).

Second, and more importantly, a careful reading of US02/0109841 will show that the USPTO reads the reference out of context. Note that the passage relied upon by the USPTO, at the end of paragraph 0046 of US02/0109841, refers to

> collecting the emission light from the sample in a microtiter well or on a two dimensional surface such as a glass microscope slide, polyacrylamide gel, silicon microarray or other solid surfaces.

The reference is discussing the collection of emission light which is emitted from a surface of the material being analyzed (or from a surface of a medium containing the material being analyzed). In this context, a polyacrylamide gel may be regarded as a "surface": this is where emission occurs, and thus for spectrophotometric purposes, the gel (or any other material being analyzed) effectively amounts to a surface. However, in the context of nucleic acid hybridization, a gel acts as a three-dimensional matrix and would be regarded as such. Note that even US02/0109841 essentially admits as much, since in paragraph 0038 – which discusses the location of the materials being analyzed, rather than the location from which analysis signals are emitted – it is stated that:

> Most fluourescence detection involves examination of specimens that are in a liquid phase.... the liquid can be trapped ***in*** a two-dimensional polyacrylamide or agarose gel.

(Emphasis added.) In other words, when read in context, it is clear that nucleic acids are *within* a thin gel, and light *from the surface* of the gel can be spectrophotometrically analyzed.

3.f. <u>The Art of Record Also Fails to Teach or Suggest the Recited Intercalating Dye</u>

Apart from the foregoing, the recited intercalating agent is a further basis for the unobviousness of the claimed invention. The Examiner alleges that the substitution of the fluorescent labels used in *Drobyshev et al* for the SYBR Green I marker used in examples in *Wittwer* would represent an obvious modification of the *Drobyshev et al* approach. However, *Drobyshev et al* does not mention or suggest the possibility of replacing the fluorescent label with any intercalating agent for the determination of the Tm of the probe/target complex. An artisan would, in fact, foresee various problems in using such intercalating agents in such a method. The double stranded sequence in *Drobyshev et al* (10bp: 1 turn of DNA helix) is considerably shorter than in *Wittwer* (110bp Example 2) and it would not be clear to an artisan whether an intercalating agent, in particular a double strand specific intercalating agent, would bind to such a short

sequence or whether the sensitivity provided, in the event of successful binding, would be sufficient for detection over the background signals from inter-target interactions. For allele discrimination, sudden and consistent denaturation is required to produce detectable differences in melting curves between wild-type and mismatch probe/target complexes. The effect of an intercalating agent as opposed to a labeled probe, on the denaturation characteristics of the probe/target complex, would not be predictable to an artisan prior to the present invention. Replacing labeled target nucleic acid molecules with an intercalating agent would therefore involve considerable additional experimentation with no guarantee that the intercalating agent would work effectively in this context. Given that the signal might be enhanced in accordance with the teachings of *Drobyshev et al* by incorporating dUTP-fluorescein into the RNA, instead of simply adding a single 3' fluorescein label, an artisan would not be motivated to attempt to employ intercalating agents.

Furthermore, motivation to attempt to employ an intercalating agent would not be obtained from the teachings of *Wittwer*. In *Wittwer*, the melting curves using the double strand specific dye are used to discriminate between different PCR products (i.e. double stranded DNA molecules of completely different sequences) in Examples 14 to 20. However, *Wittwer* goes on to state: "when sequence specific detection and quantification are desired, resonance energy probes can be used instead of double-strand-specific DNA dyes" (column 42 lines 52-55). Accordingly, Examples 21 to 24, which show single base mismatch detection, employ two DNA bound FRET fluorophores not double-strand-specific DNA dyes. A person of ordinary skill is thus taught by *Wittwer* that such dyes are not suitable for allelic discrimination and that a pair of DNA-bound FRET fluorophores should be used when single base mismatch detection is required. Were a person of ordinary skill to attempt to modify the method of *Drobyshev et al* in accordance with the teaching of *Wittwer*, he would therefore seek to employ pairs of DNA-bound FRET fluorophores, and would not employ a double-strand-specific DNA dye. A method so modified would not fall within the present claims.

The Examiner further alleges that *Wittwer* teaches a method which comprises monitoring the formation or dissociation of a complex consisting of (a), (b) and (c). This is not the case.

Complexes which form as part of PCR necessarily comprise the additional component of a thermostable DNA polymerase enzyme, which tracks along the template strand synthesizing the second strand. Notwithstanding the presence or absence of thermostable DNA polymerase, the PCR products analyzed in the melting curve analysis shown for example in Figure 37 of *Wittwer* are double stranded DNA molecules and components (a) and (b) of the complex of the present claims are not present. There is therefore no teaching in either *Drobyshev et al* or *Wittwer* of a complex as set out in the present claims.

The presently claimed method relates to the hybridization of an allele specific oligonucleotide probe in solution with a single DNA strand of a double stranded DNA (i.e. a target molecule) bound to a solid surface. This configuration is not found in *Drobyshev et al*, where the oligonucleotide probe is immobilized and the target RNA is free in solution, or in *Wittwer*, which fails to teach immobilization of either probes or target. The presently claimed arrangement of probe and target would not be obvious to an artisan from the teachings of *Drobyshev et al* and *Wittwer*, taken either individually or together.

For the reasons explained above, the combination of *Drobyshev et al* and *Wittwer* fails to teach a complex consisting of features (a), (b) and (c) of the present claims, nor does it provide any expectation that hybridization characteristics of this complex, as monitored by a double-strand specific dye, might be suitable (in terms of signal strength and signal/background ratio) for routinely discriminating with high accuracy between different alleles of a nucleic acid sequence in a dynamic (real-time) assay format. The present claims are therefore unobvious over *Drobyshev et al* in the light of *Wittwer*, and withdrawal of the §103(a) rejections is requested.

4. Section 3 of the Office Action: Rejection of Claims 1-8, 10-21, 23-34, 36-47, and 49-52 under 35 USC §103(a) in view of *Drobyshev et al* (Gene (1997) 188:45-52, U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 6,048,690 to *Heller et al*

For the reasons described above, the combined teachings of *Drobyshev et al* and *Wittwer et al* are deficient in teaching the features of the presently claimed methods. *Heller et al* does not remedy these deficiencies and the combination of *Drobyshev et al*, *Wittwer* and *Heller et al* also

fails to teach the features of the presently claimed methods. *Heller et al* describes the use of an electric field to induce perturbations in the fluorescence of a labeled probe hybridized to a oligonucleotide which is bound to a chip via a biotin/streptavidin interaction. However, the present methods employ heating to denature nucleic acid complexes. Immobilization via a non-covalent protein/cofactor interaction such as biotin/streptavidin would be considered to be unsuitable by an artisan for use at high temperature because of the possibility of streptavidin denaturation. The recognition by the present inventors that the biotin/streptavidin interaction was in fact suitable for attachment of nucleic acids at high temperatures and would provide sufficient binding capacity for dynamic reading was unexpected at the time of the invention. Since the combination of *Drobyshev et al* in view of *Wittwer et al*, and further in view of *Heller et al*, fails to suggest the features of the claimed methods, withdrawal of the rejections is requested.

5. Section 4 of the Office Action: Rejection of Claims 1-6, 8-19, 21-32, 34-45, and 47-52 under 35 USC §103(a) in view of *Drobyshev et al* (Gene (1997) 188:45-52), U.S. Patent 6,174,670 to *Wittwer*, and U.S. Patent 5,789,167 to *Konrad et al.*

For the reasons described above, the combined teachings of *Drobyshev et al* and *Wittwer* are deficient in teaching the features of the presently claimed methods. *Konrad et al* does not remedy these deficiencies, and the combination of these teachings with the teaching of *Konrad et al* also fails to teach the features of the presently claimed methods.

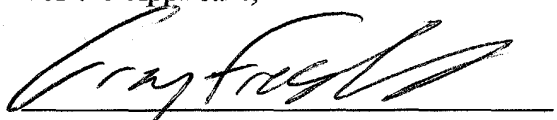
DNA buffer systems generally use high salt concentrations in order to stabilize DNA. This is evidenced on column 14 line 64 of *Konrad et al*, in which hybridization is performed in 300mM NaCl. The present inventors have recognized that in the presence of duplex specific dyes such as SYBR Green 1, high levels of salt lead to displacement of the dye and thus desensitization of the assay. For this reason, salt levels of around 50mM or less are preferred in the present methods, with a maximum upper limit of 200mM (first paragraph of page 13 of the present specification). A buffer which combines HEPES and low salt is not taught in either *Konrad, Drobyshev et al* or *Wittwer* and a person of ordinary skill would not expect such a buffer to be

useful for nucleic acid hybridization. This feature of the present claims is therefore unobvious over the combined disclosures of these documents, and withdrawal of the rejection is requested.

6. In Closing

If any questions regarding the application arise, please contact the undersigned attorney. Telephone calls related to this application are welcomed and encouraged. The Commissioner is authorized to charge any fees or credit any overpayments relating to this application to deposit account number 18-2055.

For the Applicant,



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ATTACHMENTS:

- Amendment Sheet ("Marked-Up" Copy) Showing Changes to Application
- *Kochinsky & Mirzabekov* (2002) Human Mutation 19: 343-360
- *Livshits & Mirzabekov* (1996) Biophysical J. 71 2795-2801
- *Peterson et al*, J. Am. Chem. Soc. (2002) 124 14601-14607
- *Livshits et al* (1994) J. Biomol. Struct. Dyn. 11 783-795
- Mirzabekov Declaration
- Kwok Declaration

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.:	09/755,747	Group Art Unit:	1637
Filing Date:	January 5, 2001	Examiner:	Fredman, J.
Applicants:	Brookes et al.	Atty. Docket:	40225.000
Title:	**DETECTION OF NUCLEIC ACID POLYMORPHISM**		

AMENDMENT SHEET ("MARKED-UP" COPY) SHOWING CHANGES TO APPLICATION (37 CFR §§1.121(b)(1)(iii); (c)(i)(ii))

(To Accompany Response to September 11, 2002 Office Action)

In accordance with 37 CFR §§1.121(b)(iii) and (c)(ii), following are the amendments made to the specification and/or claims of the above-noted application.

- All deletions are indicated by brackets **[like so]** and all additions are indicated by underlining **like so**.
- The additions and deletions are made with respect to the application as it is understood to exist prior to entry of this amendment (i.e., any amendments are made with respect to the previous version).
- While 37 CFR §§1.121(b)(1)(iii) and (c)(1)(ii) does not require that new and canceled paragraphs and claims be supplied on this "marked-up" copy, such new additions and cancellations are nevertheless provided below to aid the reviewer's understanding.

IN THE CLAIMS:

Claims 6, 19, 32, and 45 are canceled without prejudice to further prosecution of these claims in one or more continuing applications.

Claims 1, 7, 9, 11, 14, 20, 22, 24, 27, 33, 35, 37, 40, 46, 48, and 50 are amended as follows:

1. **[TWICE AMENDED]** A method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of:
   (a) a single DNA strand of a double stranded DNA **of at least 40 base pairs** containing the locus of a variation bound to a solid surface,
   (b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,
   (c) **an intercalating dye [a marker]** specific for the DNA duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the DNA duplex,

   which method comprises:
   (1) continually measuring an output signal indicative of interaction of the **dye [marker]** with duplex formed from the strand (a) and probe (b), and
   (2) recording the **temperature [conditions]** at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

7. **[AMENDED]** A method according to claim **1 [6]**, in which **the single strand is bound to the solid surface [attachment is]** by a biotin/streptavidin type interaction.

9. **[AMENDED]** A method according to claim 8, in which the buffer solution is Hepes buffer **having a salt concentration less than 200 mM**.

11. **[AMENDED]** A method according to claim 1, in which **[the single strand is derived from a]** double stranded DNA **is a** product of PCR amplification of a target sequence.

14. **[TWICE AMENDED]** A method of detecting DNA variation which comprises bringing together:

(a) a single DNA strand of a double stranded DNA **of at least 40 base pairs** containing the locus of a variation bound to a solid surface,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,

(c) **an intercalating dye [a marker]** specific for the DNA duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

thereby forming a complex consisting of the components (a), (b) and (c), wherein the components (a), (b), and (c) are brought together under conditions in which either

(i) the component (a) hybridizes to component (b) and the complex is formed with component (c), or

(ii) the components (a) and (b) do not hybridize and the complex with component (c) is not formed,

(2) thereafter steadily and progressively adjusting the **temperature [conditions of the environment]**, respectively, either

(i) to denature the formed DNA duplex and cause dissociation of the complex, or

(ii) to cause formation of the DNA duplex and resulting complex,

(3) continually measuring an output signal indicative of the extent of hybridization of (a) and (b) and resulting complex formation with (c), and

(4) recording the temperature at **[conditions in]** which a change of output signal occurs which is indicative of, respectively,

(i) dissociation of the complex, or

(ii) formation of the complex.

20. **[AMENDED]** A method according to claim **14 [19]**, in which **the single strand is bound to the solid surface [attachment is]** by a biotin/streptavidin type interaction.

22. **[AMENDED]** A method according to claim 21, in which the buffer solution is Hepes buffer **having a salt concentration less than 200 mM**.

24. **[AMENDED]** A method according to claim 14, in which the **[single strand is derived from a]** double stranded DNA **is a** product of PCR amplification of a target sequence.

27. **[TWICE AMENDED]** A method of detecting DNA variation which comprises:
(1) forming a complex consisting of:
(a) a single DNA strand of a double stranded DNA **of at least 40 base pairs** containing the locus of a variation bound to a solid surface,
(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridized to the single strand (a) to form a duplex, and
(c) **an intercalating dye [a marker]** specific for the DNA duplex structure of (a) plus (b) and which reacts uniquely when interacting within the DNA duplex, and
(2) continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex while steadily increasing the **temperature [denaturing environment containing the complex]**,
(3) recording the **[conditions]** **temperature** at which a change in reaction output signal occurs **[(herein termed the denaturing point)]** which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

33. **[AMENDED]** A method according to claim **27** **[32]**, in which **the single strand is bound to the solid surface** **[attachment is]** by a biotin/streptavidin type interaction.

35. **[AMENDED]** A method according to claim 34, in which the buffer solution is Hepes buffer **having a salt concentration less than 200 mM**.

37. **[AMENDED]** A method according to claim 27, in which the **[single strand is derived from a]** double stranded DNA **is a** product of PCR amplification of a target sequence.

40. **[TWICE AMENDED]** A method of detecting DNA variation which comprises:
(1) bringing together:
(a) a single DNA strand of a double stranded DNA **of at least 40 base pairs** containing the locus of a variation bound to a solid surface,
(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,
(c) **an intercalating dye [a marker]** specific for the DNA duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,
the components (a), (b) and (c) being brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridize;
(2) steadily adjusting the **temperature [conditions of their environment]** to cause formation of the duplex and resulting complex consisting of components (a), (b), and (c), and
(3) measuring an output signal indicative of the occurrence of hybridization of (a) and (b) (herein termed the annealing point).

46. **[AMENDED]** A method according to claim **40 [45]**, in which **the single strand is bound to the solid surface [attachment is]** by a biotin/streptavidin type interaction.

48. **[AMENDED]** A method according to claim 47, in which the buffer solution is Hepes buffer **having a salt concentration less than 200 mM**.

50. **[AMENDED]** A method according to claim 40, in which the **[single strand is derived from a]** double stranded DNA **is a** product of PCR amplification of a target sequence.

New claims 53-56 are added as follows:

53. **[NEW]** A method according to claim 4 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

54. **[NEW]** A method according to claim 17 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

55. **[NEW]** A method according to claim 30 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

56. **[NEW]** A method according to claim 43 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.